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Molecular cloning and nucleotide sequence of a new P450 gene, *CYP319A1*, from the cattle tick, *Boophilus microplus*

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Abstract

We have isolated and sequenced a novel P450 gene (*CYP319A1*) from the cattle tick, *Boophilus microplus*. The *CYP319A1* cDNA encodes a protein of 531 amino acids with an estimated molecular weight of 60.9 k. It contains all highly conserved motifs characteristic of P450 enzymes. Comparison of deduced amino acid sequence with other CYP members shows that the CYP319A1 is more closely related to CYP4 family, but its overall identity to the CYP4 family is less than 40%. Therefore, it was assigned to a new P450 family by the P450 nomenclature committee. A pseudogene which shares high homology with the *CYP319A1* was identified. Analysis of genomic sequence of the pseudogene indicated that the pseudogene contains two additional DNA inserts in the coding region, which disrupt the open reading frame. RT-PCR analysis showed that CYP319A1 is expressed in both susceptible and acaricide-resistant ticks. Published by Elsevier Science Ltd.

Keywords: cDNA; Cytochrome P450; Pseudogene

1. Introduction

P450 enzymes (mixed function oxidases, cytochrome P450 monooxygenases) are a complex family of heme-containing enzymes found in most organisms. P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen atom to the substrate. In insects, the diverse functions of P450 enzymes range from the synthesis and degradation of ecdysteroids and juvenile hormones to the metabolism of xenobiotics (for review, see Feyereisen, 1999). P450 enzymes play important roles in adaptation of insects to toxic compounds in their host plants and are involved in metabolism of all commonly used insecticides. P450 enzymes metabolize organophosphorus insecticide compounds to more active toxicants by activation of a P=S bond to a P=O bond (Feyereisen, 1999; Sams et al., 2000). However, in general, P450 enzymes mediate metabolic detoxification of other insecticides, particularly pyrethroids. Over-expression of P450 members of CYP4, CYP6, and CYP9 families has been observed in insecticide-resistant strains of several insects (Cariño et al., 1994; Feyereisen et al., 1995; Tomita et al., 1995; Wang and Hobbs, 1995; Brun et al., 1996; Scott et al., 1996; Pittendrigh et al., 1997; Rose et al., 1997).

Recently, a new nomenclature was introduced to designate all gene members of the P450 super-family with a *CYP* prefix, followed by a numeral for the family, a letter for the subfamily, and a number for the individual gene (Nelson et al., 1996; Feyereisen, 1999). This system arbitrarily defines that members of a family share >40% identity in amino acid sequence, and members of a subfamily share >55% identity (Feyereisen, 1999).

The southern cattle tick, *Boophilus microplus*, is one of the most important pests of cattle in tropical and subtropical countries because of its transmission of the protozoan parasites *Babesia* and *Anaplasma* that cause cattle diseases. As is true with other arthropod pests, *B. microplus* has developed resistance to various pesticides. We undertook efforts to clone the P450 genes because there is evidence that P450 enzymes might be involved in some of the resistance (Miller et al., 1999). There has been only one P450 gene (*CYP4W1*) cloned recently

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from the cattle tick, *B. microplus* (Crampton et al., 1999), and it is the only P450 gene reported in any arachnid. We report here a new P450 gene (*CYP319A1*) and a pseudogene from *B. microplus*.

2. Materials and methods

2.1. Animals

B. microplus larvae were obtained from the Cattle Fever Tick Research Laboratory, USDA-ARS, Mission, TX, where all strains were colonized by standard mass rearing procedures (Miller et al., 1999). Six strains used in this study were Gonzalez (G), Ramireño (R), Tuxpan [T, also referred to as the Mexican organophosphate (OP)-resistant strain], Coatzacoalcos (Cz, also referred to as the Mexican pyrethroid-resistant strain), Corrales (C), and San Felipe (S). Strains G and R are susceptible to both OP and pyrethroid acaricides. Strain T is OP-resistant, but susceptible to pyrethroids. Strain Cz has a moderate resistance to pyrethroids. Both strains C and S are highly resistant to pyrethroids and we have identified a mutation in the sodium channel gene in these strains (He et al., 1999). Strains G and R have been maintained in the absence of any acaricidal pressure during the colonization process, whereas the resistant strains (T, Cz, C, and S) have been selectively pressured through multiple generations with appropriate acaricides to maintain or increase the resistance level of each strain. Only unfed larvae were used in this study to avoid potential contamination by host DNA.

2.2. Isolation of mRNA and synthesis of cDNA

Total RNA was isolated from tick larvae using TRIzol Reagent (Life Technologies, Gaithersburg, MD). Poly A^+ mRNA was purified from the total RNA using Oligotex resin (Qiagen, Santa Clarita, CA). For cloning, cDNA was synthesized from mRNA (2 μg) using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA). This cDNA was primarily used for 5′- and 3′-RACE (rapid amplification of cDNA ends) to clone the full-length cDNA. For PCR estimation of gene expression, first-strand cDNA was prepared using the SuperScript Preamplification System (Life Technologies).

2.3. Oligonucleotide primers and PCR amplification of CYP319A1 cDNA fragment

Primers used in this study were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Two degenerate primers (DP1, 5′-ACI TTY ATG TTY RAR GGI CAY GAY AC-3′ and DP2, 5′-AAI KYY IGI CCD ATR CAR TTI CKI GG-3′: I=deoxyinosine; D=A, G, T; K=G, T; R=A, G; Y=C, T) were designed based on the conserved amino acid sequences of CYP4 family. DP1 was derived from CYP4 specific sequence motif TFMFEGHDT, while DP2 was derived from the heme-binding site sequence PRNCIGQA/T/KF (Fig. 1). PCR reactions, performed on the PTC-200 DNA Engine (MJ Research, Watertown, MA), contained cDNA (5 μl 1:50 diluted Marathon™ cDNA), 200 μM each of dNTPs, 10 μM each of degenerate primers DP1 and DP2, 0.5 μl Advantage KlenTaq Polymerase Mix, and 1× reaction buffer (Clontech) in a final volume of 50 μl. The cycling conditions were: 94°C for 2 min followed by 10 cycles of 94°C for 20 s, 65°C for 30 s with decrement of 1°C per cycle, 68°C for 2 min and 30 cycles of 94°C for 20 s, 55°C for 30 s, 68°C for 2 min and then a final extension at 68°C for 10 min. PCR products were analyzed on agarose gels. The DNA band of expected size was excised with a pipette tip, eluted in 100 μl 0.1× TE buffer by boiling 3 min in a water bath, and reamplified. Reamplified PCR products were cloned using the Zero Blunt PCR cloning Kit (Invitrogen, Carlsbad, CA). Plasmids were isolated using the alkaline miniprep procedure (Sambrook et al., 1989) and further purified by Advantage PCR-Pure Kit (Clontech).

2.4. 5′- and 3′-RACE

Amplification of 5′- and 3′-ends of cDNA (5′- and 3′-RACE) was performed according to the protocol described in the Marathon™ Amplification Kit (Clontech). Four gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers (Section 2.3). The primer pairs AP1 (adaptor primer 1, Clontech)/GSP-R1 and AP1/GSP-F1 were used for the initial 5′- and 3′-RACE, respectively (see Fig. 1 for primer sequences). Initial 5′- and 3′-RACE PCR reactions were diluted by 50-fold and a 5 μl aliquot of diluted PCR reactions was used in each of 50 μl nested-PCR reactions using primer pairs AP2/GSP-R2 and AP2/GSP-F2 for 5′- and 3′-RACE, respectively. The initial RACE PCR reactions contained the same components as in the degenerate primer PCR except that the final concentration of each primer was 0.2 μM. PCR reactions started with 94°C for 2 min followed by 5 cycles of 94°C for 20 s, 72°C for 90 s, 5 cycles of 94°C for 20 s, 70°C for 90 s, and 25 cycles of 94°C for 20 s, 68°C for 90 s and a final extension at 68°C for 10 min. Nested-PCR reactions contained the same components as in the initial PCR except that diluted initial PCR products were used instead of the Marathon™ cDNA as the template. The cycle conditions for the nested-PCR were 94°C for 1 min followed by 25 cycles of 94°C for 20 s, 68°C for 90 s and a final extension at 68°C for 10 min. RACE PCR products were purified, cloned, and sequenced as described. The full-length cDNA of *CYP319A1* was amplified using two

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *CYP319A1* cDNA. Polyadenylation signal AATAAA is shown in bold italic lowercase letters. Star denotes translational stop codon. White letters in black background are nucleotides mutated in pseudogene *CYP319A1P*. Shaded amino acid residues are the regions of predicted transmembrane sequences. Boxed nucleotides are the 70-bp sequence deleted in the pseudogene *CYP319A1P* cDNA. Underlined amino acid sequences were used for designing degenerate primers. Lines with arrow indicate nucleotide sequences of gene specific primers used for the 3′- and 5′-RACE and semi-quantitative PCR: 1, GSP-F3; 2, GSP-R3; 3, GSP-F2; 4, GSP-F1; 5, GSP-R2; 6, GSP-R1.

GSP complementary to 5′- and 3′-ends of the cDNA sequence.

2.5. Amplification of genomic DNA

Genomic DNA was isolated from tick larvae according to the method described by Sambrook et al. (1989). Primers based on the cDNA sequence were used for PCR amplification. PCR products amplified from the genomic DNA were compared with the cDNA to determine the location and size of any introns that may be present.

2.6. Semi-quantification of the CYP319A1 expression in different tick strains

The expression of *CYP319A1* in larvae from different tick strains was examined by semi-quantitative PCR. Two primers (Fig. 1, primers GSP-F3 and GSP-R3) were selected to amplify the cDNA fragment such that the PCR product from *CYP319A1* cDNA can be differentiated from the pseudogene cDNA, which is 70 bp shorter. Thus, the expression of the functional gene can be examined. A pair of primers derived from tick β-actin cDNA (unpublished data, 5′-TCC TCG TCC TGG AGA AGT CGT AC and 5′-CCA CCG ATC CAG AGG GAG TAC TTC) was used to amplify a fragment of β-actin cDNA as the reference for estimation of *CYP319A1* expression. First-strand cDNA from six tick strains were synthesized from 0.5 μg mRNA. A 5 μl aliquot of the 1:10 diluted cDNA was used in each of the 50 μl PCR reactions. PCR cycles were predetermined within the exponential phase. A 35-cycle PCR was selected for both β-actin and CYP319A1 assays. Cycling conditions were 94°C for 1 min followed by 35 cycles of 94°C for 20 s and 68°C for 1 min for the β-actin and 94°C for 1 min followed by 35 cycles of 94°C for 20 s, 65°C for 20 s, and 68°C for 40 s for the *CYP319A1*. Both PCR reactions were completed with a final extension at 68°C for 5 min.

2.7. Sequence analysis and phylogenetic tree construction

DNA sequence was determined using an ABI-PRISM automated DNA sequencer (Model 377) at the DNA Sequencing and Oligo Lab, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX. Sequence analyses and data searches were performed using programs from the BCM Search Launcher (http://www.hgsc.bcm.tmc.edu/SearchLauncher, Human Genome Center, Baylor College of Medicine, Houston, TX) on the Internet. A phylogenetic tree was constructed using the Phylogenetic Tree Prediction program from the Genebee Service (Belozersky Institute of Physico-Chemical Biology, Moscow State University, Russia, http://www.genebee.msu.su/genebee.html) on the Internet.

3. Results and discussion

3.1. cDNA and deduced amino acid sequence of the CYP319A1

Gene cloning by PCR using degenerate primers derived from conserved amino acid sequences from other species has proven to be a powerful method to obtain related DNA sequences from the target species. Although the P450 super-family has a very divergent sequence and the overall homology may be less than 40% even within the same family, particularly in insects (Wang and Hobbs, 1995), there are function-critical sequence motifs preserved during evolution. For the CYP4 family, in addition to the heme-binding sequence motif F××G×××C×G universal among CYP enzymes (Feyereisen, 1999), the characteristic motif of EVDTFMFEGHDTT is conserved (Fig. 2). In this study, two degenerate primers derived from these two conserved amino acid sequences in the CYP4 family were used to amplify a P450 sequence from the tick cDNA by PCR. A cDNA fragment of ~450 bp was amplified by PCR and the product was purified from the agarose gel and cloned. The DNA sequence was determined and then compared to sequences in the GenBank database. The amplified sequence had a high similarity to numerous *CYP* gene sequences. Full-length cDNA sequence was obtained by 5′- and 3′-RACE. The 3′-RACE reaction produced a single DNA band observed following agarose gel electrophoresis and the DNA fragment was purified and directly sequenced. No discrepancies were observed. However, the sequence electrophoretogram from the 5′-RACE product indicated that there were mixed DNA sequences in the reaction. After cloning the 5′-RACE product and sequencing several clones, three very closely related cDNA sequences were obtained, one functional gene cDNA sequence (AF261080) and two pseudogene cDNA sequences (AF261081 and AF261082). These two pseudogene cDNA sequences differ only at the 5′-end of the non-coding region.

The functional gene cDNA sequence contains an open reading frame with 5′- and 3′-untranslated regions (Fig. 1). The putative polyadenylation signal AATAAA was identified at 28-bp upstream of the poly (A) tail. The open reading frame encodes a protein of 531 amino acids with a calculated molecular weight of 60.9 k. Analysis of the deduced amino acid sequence indicates a CYP signature motif (the heme-binding sequence F××G×××C×G) and a motif (EVDTFMFEGHD) characteristic of the CYP4 family (Fig. 2). However, the overall identity of this deduced amino acid sequence to other CYP4 members is less than 40%. The sequence was submitted to the P450 nomenclature committee and was assigned to a new CYP family, CYP319A1 (David R. Nelson, personal communication). The CYP319A1 sequence shares 39% identity with another recently cloned tick P450 member, CYP4W1 (Crampton et al., 1999), which is the highest compared to other CYP4 members. Analysis with the SOSUI program for protein secondary structure prediction (Hirokawa et al., 1998) indicates that the CYP319A1 is a membrane protein with

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                 320                      393      447          466
tCYP319A1  ...LTTDDIREEVDTFMFEGHDTT...KECQRL...DPEVFDPDR...PFSAGPRNCIGQ
tCYP4W1    ...FTEEHVREEVDTFMFEGHDTT...KESQRL...KPDEFRPDR...PFSAGPRNCIGQ
hCYP4B1    ...LSDADLRAEVDTFMFEGHDTT...KESFRL...DPEVFDSLR...PFSAGPRNCIGQ
hCYP4A     ...LSDKDLRAEVDTFMFEGHDTT...KEALRL...NPEVFDPFR...PFSGGSRNCIGK
dCYP4D1    ...LSNLDIREEVDTFMFEGHDTT...KETLRM...EPNSFKPER...PFSAGPRNCIGQ
cCYP4C1    ...MSDTDIREEVDTFMFEGHDTT...KESLRL...NPEAFNPDN...PFSAGPRNCIGQ
hCYP1A1    ...LSDEKIINIVLDLFGAGFDTV...LETFRH...NPSEFLPER...IFGMGKRKCIGE
rCYP2A1    ...FHMKNLVMTTLSLFFAGSETV...NEIQRF...SPKDFDPQN...PFSTGKRFCLGD
rCYP3A1    ...LSDMEITAQSIIFIFAGYEPT...NETLRL...EPEEFRPER...PFGNGPRNCIGM
mCYP6A1    ...LTFNELAAQVFVFFLGGFETS...NETLRK...NPEEFDPER...GFGDGPRNCIGM
Conserved AA  ..................T.....ExxR.....PxxFxP......FxxGxxxCxG.
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Fig. 2. Comparison of CYP319A1 to members of other CYP families in regions conserved in P450 enzymes. Shaded amino acid residues are absolutely conserved in P450 enzymes and the motif (EVDTFMFEGHD) reserved in CYP4 family. Numbers are in reference to CYP319A1 sequence. First italic letters of the names indicate sources of the P450s: *t*, tick; *h*, human; *d*, Drosophila; *c*, cockroach; *r*, rat; *m*, *Musca domestica*.

two transmembrane domains located near the N-terminus. The primary transmembrane domain contains amino acid residues 3–25 and the secondary one contains residues 49–71. These highly hydrophobic N-terminal regions are present in most P450 enzymes and serve as the signal peptide for co-translational insertion of the protein into the target membrane (Pernecky and Coon, 1996). Other important amino acid residues conserved in P450 enzymes (Fig. 2) were also identified in the CYP319A1 using the Multiple Alignment Program (MAP, http://www.hgsc.bcm.tmc.edu/SearchLauncher). These include threonine 339 which is located in the middle of the I-helix in the tertiary structure of P450 enzymes and is thought to be important in the activation of molecular oxygen, the motif of E××R (394–397) for salt bridge formation in the mature protein, and the consensus sequence P××F×PE (448–454) of a microsomal P450 (Graham-Lorence and Peterson, 1996). These structural futures indicate that the CYP319A1 is likely a microsomal P450 enzyme and is closely related to the CYP4 family.

3.2. *A pseudogene evolved from the* CYP319A1

Pseudogenes are DNA sequences that have significant homology to functional genes, but which contain no promoters or mutations, deletions, and insertions within their protein coding regions, that render their transcription silent or coding region aberrant. Most pseudogenes derive from duplicated genes formed within the gene clusters and have diverged to become inactive. However, a small number of pseudogenes, which have sequence characteristics of intron-less and oligo-A tracts at their 3′-ends, have been identified recently as processed pseudogenes. These processed pseudogenes may have arisen by retroposition events by which the processed RNA was converted to cDNA by reverse transcription and then integrated into the genomic DNA (Wilde, 1986).

Pseudogenes and processed pseudogenes have been reported in several P450 families (Trottier et al., 1996; Rozman et al., 1996; Crampton et al., 1999). In this study, two cDNA sequences from the pseudogene were amplified along with the cDNA from the functional gene. These pseudogene cDNA sequences were identical except the 5′-end non-coding region, which was probably a result of an alternative-splicing event. Therefore, we have named the pseudogene *CYP319A1P* in accordance with the P450 nomenclature (Nelson et al., 1996). Comparison of the *CYP319A1P* cDNA to the *CYP319A1* cDNA sequence showed that they are highly homologous in the coding region with only three non-silent nucleotide changes and a deletion of a 70-bp sequence in the pseudogene cDNA (Fig. 1). The deletion of the 70-bp sequence has caused the shift of the open reading frame and resulted in premature termination. The truncated product contains no functionally important sequences and is likely non-functional. At first, we suspected that the truncated cDNA might be an alternative-splicing product. However, we amplified the region surrounding the deleted sequence from the genomic DNA using PCR and obtained DNA fragments (~258 and ~1800 bp) in the same reaction. DNA sequencing showed that the 258-bp fragment was identical to the functional gene cDNA sequence, while the 1800-bp DNA was found to contain two additional sequences, one (~1200 bp) was inserted at the 5′-end of the deleted fragment and the other (~320 bp) at the 3′-end (Fig. 3). Apparently, these two DNA inserts along with the 70-bp deleted region were spliced during the mRNA processing, which renders the coding region aberrant. Most pseudogenes have diverged to become transcriptionally silent after evolving from their functional parent genes (Wilde, 1986). Judging from the high level of homology of the *CYP319A1P* cDNA sequence to the functional gene and the fact that it was transcriptionally active, we conclude that this pseudogene is most likely to have arisen relatively recently. A recent study (Crampton et al., 1999) has also identified a pseudogene *CYP4W1P* from *B. micorplus*. Unlike *CYP319A1P*, the *CYP4W1P* is an intron-less processed pseudogene, but it also appears to have arisen relatively recently.

3.3. *Phylogenetic relationship with other CYP families*

The deduced amino acid sequence of CYP319A1 contains all important motifs characteristic of the P450 enzymes, particularly the CYP4 family. Using the Pairwise Sequence Alignments program (ALIGN, http://dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html), we have obtained levels of the amino acid sequence identity of CYP319A1 to selected members from other CYP families (Fig. 4). The CYP319A1 sequence was found more closely related to the CYP4W1 from the tick (Crampton et al., 1999) and other CYP4 members than families of CYP1, CYP2, CYP3, and insect CYP6. Not surprisingly, phylogenetic analysis of amino acid sequences from members of different CYP families indicates closer phylogenetic relationship of CYP319A1 with CYP4 members from ticks and other insects than from human and more distant to P450 members from other families (Fig. 4).

3.4. *Expression of* CYP319A1 *and its pseudogene in different tick strains*

CYP319A1 was first amplified from the cDNA that was made from larvae of the pyrethroid-resistant Cz strain. To investigate whether *CYP319A1* was also expressed in other tick strains, we amplified the *CYP319A1* cDNA from six different tick stains. PCR produced cDNA from both the functional gene and the

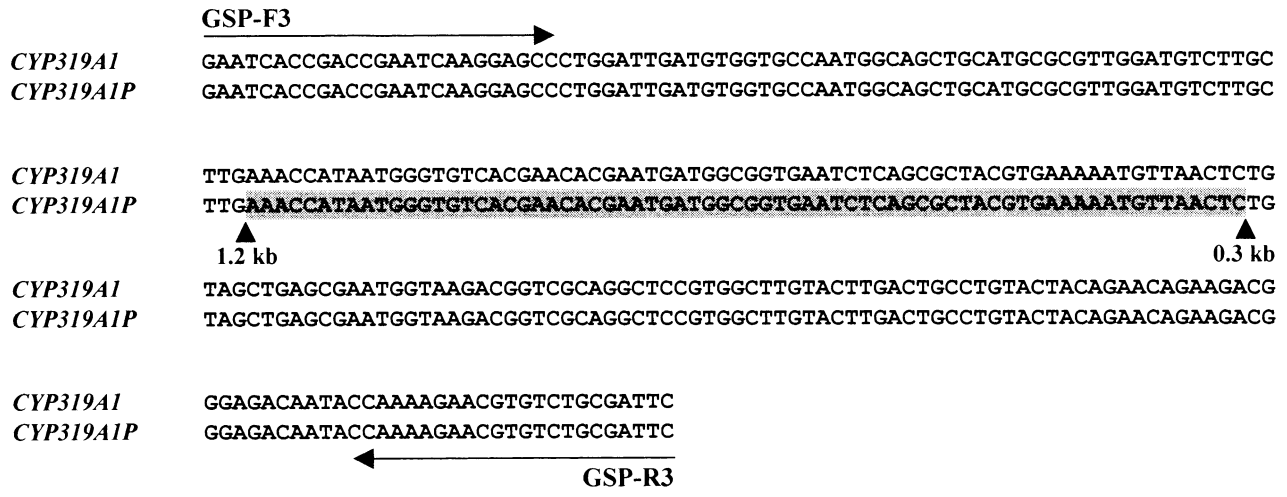

Fig. 3. Comparison of partial genomic sequences of the functional gene *CYP319A1* with pseudogene *CYP319A1P*. Lines with arrow indicate sequences of the primers used for amplification of the genomic sequences. Shaded sequence indicates the region of the pseudogene that is spliced during RNA processing. Upward arrows and numbers indicate the locations and approximate size of DNA inserts.

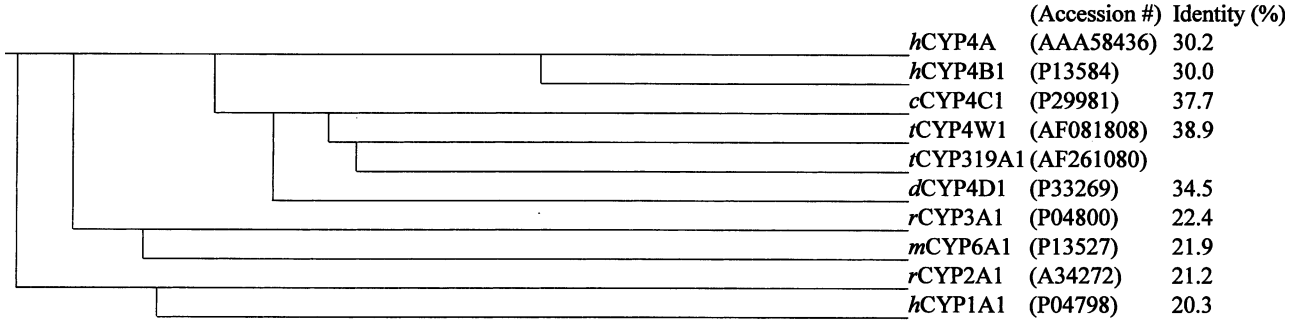

Fig. 4. Phylogenetic relationship based on amino acid sequence comparisons of P450s from various CYP families. In cluster algorithm, distance between groups of sequences is used for the setting of the branching order. This distance is the arithmetic mean of pairwise distances between elements of the two groups. GenBank accession numbers are given in parenthesis. Identity is obtained by pairwise alignment of amino acid sequence of CYP319A1 with indicated P450. Letter designation: *h*, human; *c*, cockroach; *t*, tick; *d*, fruit fly; *r*, rat; *m*, *M. domestica*.

pseudogene. We also performed PCR using a pair of β-actin gene primers on the same cDNA sources as a reference to estimate the expression level of *CYP319A1*. Results indicate that *CYP319A1* was expressed in tick larvae from all strains examined at similar levels (Fig. 5). The pseudogene *CYP319A1P* was also expressed in all strains, but at much lower levels. We do not know if CYP319A1 is involved in pesticide resistance in *B. microplus* and whether pesticide challenges will affect the level of *CYP319A1* expression since we did not treat the tick larvae used in this study. Further study will be conducted to investigate the response of *CYP319A1* to acaricide treatments. Since P450 is a very large and diverse family of enzymes in organisms that have been studied, it is probable that *B. microplus* has a large number of these enzymes. There is a possibility that some forms of P450s may be involved in tick resistance to pesticides.

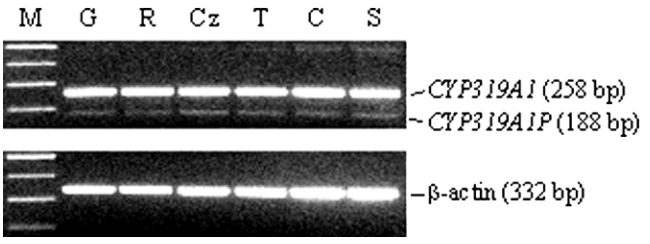

Fig. 5. Expression of *CYP319A1* and *CYP319A1P* in different tick strains. cDNA obtained with mRNA from various strains of ticks was amplified using the primer pair, GSP-F3/GSP-R3. Lane M is DNA marker. Tick strains used: G, Gonzalez; R, Ramireño; T, Tuxpan; Cz, Coatzacoalcos; C, Corrales; and S, San Felipe.

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does not constitute an endorsement of this product by the USDA, nor does it imply the recommendation of the product by the USDA to the exclusion of similar products.

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